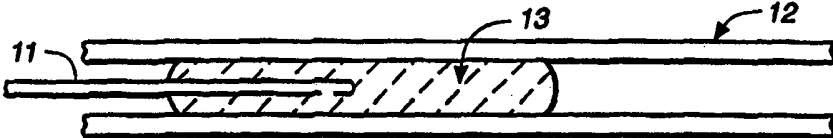




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(21) International Application Number: PCT/US98/26004 (22) International Filing Date: 8 December 1998 (08.12.98) (30) Priority Data: 08/987,287 9 December 1997 (09.12.97) US (71) Applicant: THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; Suite 350, 900 Welch Road, Palo Alto, CA 94304 (US). (72) Inventors: ZARE, Richard, N.; 724 Santa Ynez, Stanford, CA 94305 (US). DULAY, Maria, T.; 955-B La Mesa Terrace, Sunnyvale, CA 92354 (US). KULKARNI, Rajan, P.; 11632 Butterfield Street, Loma Linda, CA 92354 (US). (74) Agents: HSUE, James, S. et al.; Majestic, Parsons, Siebert & Hsue P.C., Suite 1100, Four Embarcadero Center, San Francisco, CA 94111-4106 (US).	(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>	
(54) Title: SEPARATION COLUMN CONTAINING POROUS MATRIX AND METHOD OF PACKING COLUMN  (57) Abstract A mixture of chromatographic particles and a solution of water, alcohol and metal alkoxide may be injected by means of a syringe into a capillary column as a gel. The volatile components in the gel are evaporated by means of heating and gas pressure reduction to form a porous sol-gel glass matrix attached to the inner wall of the separation channel. The pores are large enough for the passage of protons, neutral and ionic species but are too small to permit significant leaching of the chromatographic particles. The separation column so formed requires no frits to maintain the glass matrix in place in the column. Electrical potential difference and/or pressure difference may be applied to cause fluid flow in the separation column to cause electrophoretic and chromatographic separation.		

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SEPARATION COLUMN CONTAINING POROUS
MATRIX AND METHOD OF PACKING COLUMN

BACKGROUND OF THE INVENTION

The invention relates to a separation system
5 employing a column containing a porous matrix embedded
with chromatographic particles and a method of packing
a channel with the matrix to make the column.

Capillary zone electrophoresis (CZE), with its high
peak capacity (i.e., the number of peaks separated per
10 unit time), has long been proven to be an attractive
analytical technique for separating ionic species by
their electrophoretic mobilities. The separation of
neutral species via CZE, however, has remained more
problematic. To improve the separation of neutral
15 species via capillary electrophoresis, the technique of
capillary electrochromatography (CEC) has been employed,
which is a promising technique that seeks to combine the
advantages of capillary electrophoresis and
chromatography as described in the article by M.T. Dulay
20 et al. in *Chromatogr. A.*, 725 (1996) pp. 361-365).

In CEC, the separation of uncharged analytes is
based on partitioning of chromatographic particles such
as octadecylsilica, while the separations of charged
analytes are based on both partitioning and
25 electrophoretic mobility. Existing techniques for the
preparation of packed capillary columns are based on
either a slurry packing method or an electrokinetic
packing method of small-bore capillary columns. The
electrokinetic packing method may be more advantageous
30 than a slurry packing method for the preparation of

packed capillary columns with micron-sized inner diameters. Disadvantages of the electrokinetic packing method include the limited choices of chromatographic phases (i.e., only charged particles can be used) and
5 the need for both inlet and outlet frits to prevent the chromatographic particles from leaving the capillary column. This causes the columns to be difficult and time consuming to make.

It is therefore desirable to provide a separation
10 column with improved characteristics and that are easy to make.

SUMMARY OF THE INVENTION

One aspect of the invention is directed towards a separation column comprising a separation channel having
15 a channel wall and a separation medium in the channel. The medium includes a porous matrix attached to the channel wall and chromatographic particles embedded in the matrix forming a packed channel. The channel has no frit therein adjacent to the separation medium.

20 Another aspect of the invention is directed towards an apparatus for separating a sample into its components, comprising a separation channel having a channel wall; a separation medium in the channel, and means for causing a fluid containing a sample present in
25 a channel to flow and the sample to separate. The medium includes a porous matrix attached to the channel wall and chromatographic particles embedded in the matrix, forming a packed channel. The channel has no frit therein adjacent to the separation medium.

30 One more aspect of the invention is directed towards a method for making a separation column, comprising introducing a mixture of chromatographic particles and a solution of a monomer and a cross-linking reagent into a separation channel, such channel

having a wall; and causing the mixture to form a porous matrix attached to the channel wall with said particles embedded therein.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Fig. 1 is a partially cross-sectional and partially schematic view of a section of a separation column along a longitudinal axis of the column and of a needle portion of a syringe for injecting a mixture of chromatographic particles and a solution of water, alcohol and a metal alkoxide into the column to
10 illustrate a preferred embodiment of the invention.

 Fig. 2 is a cross-sectional view of a portion of the column of Fig. 1 along a longitudinal axis of the column, where the volatile components in the mixture
15 injected into the column have been removed to form a porous sol-gel with chromatographic particles embedded therein to illustrate the invention.

 Figs. 3A and 3B are cross-sectional views of the separation column shown in Fig. 2 along the line 3A, 3B
20 - 3A, 3B in Fig. 2 to illustrate two different embodiments of the separation column.

 Fig. 4 is a partially schematic and partially cross-sectional view of a separation system employing the separation column of Fig. 2 to illustrate a
25 preferred embodiment of the invention.

 Fig. 5 is a schematic view of a system for making the separation column of Fig. 2.

 Fig. 6 is a cross-sectional view of a portion of a separation column along a longitudinal axis of the
30 column to illustrate another embodiment of the column and an alternative method for packing the column.

 For simplicity in description, identical components are labeled by the same numerals in this application.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the preferred embodiment, this invention employs a metal alkoxide sol-gel process. The metal alkoxide sol-gel process is a method of preparing metal oxide glasses by hydrolyzing a solution of water, alcohol, and a metal alkoxide source. The sources of these metal oxides, or silanes, are the alkoxy compounds of type $R_nSi(OR')_{4-n}$ as described by C.J. Brinker et al. in *Sol-Gel Science*, Academic Press, Inc., New York, NY, 1990. The most commonly used of these compounds is tetraethylorthosilicate (TEOS, $Si(OC_2H_5)_4$), although other compounds such as titanates, and zirconates may also be used for this invention. As these substances polymerize, gelation of the solution occurs. If the volatile solvents in the wet gel are allowed to evaporate, the gel shrinks and hardens, creating a hard porous glass.

Since sol-gel glasses are formed from solution, other molecules can be embedded inside the pores or inside the cavities created. When the solvent evaporates from the gel, the glass that is created is porous. The porosity of the glass allows for the diffusion of protons (and other neutral or ionic species) through the channels. These pores, however, must be large enough to allow species diffusion, but small enough that significant amounts of chromatographic materials cannot leave the xerogel matrix.

Fig. 1 is a partially cross-sectional and partially schematic view of a portion of a separation column 12 along a longitudinal axis of the column and a needle portion 11 of a syringe (not shown) injecting a mixture 13 of chromatographic particles and a solution to illustrate the preferred embodiment of the invention. As shown in Fig. 1, a needle portion 11 is used to inject into a channel within a tube 12, a mixture 13 of chromatographic particles in a solution of water, alcohol and a metal alkoxide. This mixture forms a wet

gel 13 in tube 12. After the volatile solvents in the wet gel 13 have evaporated, the gel 13 shrinks and hardens, creating a hard porous glass 13' shown in Fig. 2.

5 Fig. 2 is a cross-sectional view of column 12 along a longitudinal axis of the column and the hard porous glass 13' in the column resulting from evaporation of the volatile solvents in the wet gel 13 of Fig. 1. As shown in Fig. 2, glass 13' includes pores 4 and
10 chromatographic particles 6 embedded therein. As noted above, the porosity of the glass 13' allows for the diffusion of protons and other neutral or ionic species, but small enough that significant amounts of chromatographic particles 6 cannot leave the glass
15 matrix 13'. Glass 13' forms a secure bond to the inner wall of the tube 12, so that no frit is required adjacent to the glass 13' to maintain the matrix glass 13' in place in tube 12. While pores 4 are big enough to allow diffusion of protons, neutral and ionic
20 species, they are too small for most of the chromatographic particles 6. This prevents leaching of the particles when a fluid is passed through the glass matrix. Thus, when a sample is carried by fluid through glass 13', the sample components will interact with the
25 chromatographic particles 6 and become partitioned or separated.

Tube 12 can have many different cross-sections, such as a circular cross-section shown in Fig. 3A, the cross-section being normal to a longitudinal axis of the
30 tube, shown as tube 12'. Alternatively, tube 12 can have an elongated cross-section as shown in Fig. 3B, where the tube is formed by two flat plates 12" where the tube is formed by sealing the adjacent edges of the two plates by means of an adhesive 15. Such and other
35 cross-sections are possible for tube 12 and are within the scope of the invention. In some embodiments, the

internal dimension of tube or channel 12 may range from about 5 to about 3,000 microns. Where tube 12 is a capillary, its internal dimension may range from about 5 to about 300 microns.

5 Chromatographic particles 6 may comprise uniformly sized particles of the same type, or a mixture of different types of particles of different sizes. Preferably, the particles are of dimensions greater than 0.2 microns.

10 Fig. 4 is a partially schematic and partially cross-sectional view of a separation system employing a column 12 to illustrate the preferred embodiment of the invention. As shown in Fig. 4, the inlet end 12a of the column is immersed in an electrolyte buffer 14 contained
15 in a reservoir 16. The outlet end 12b is immersed in an electrolyte buffer in reservoir 18. An electrical potential of several kilovolts is applied by high voltage source 20 through electrodes 30 between
20 electrolyte buffer 14 in input and output reservoirs 16, 18, causing a potential difference and electric field along the column 12. Such potential difference causes an electroosmotic flow in tube 12 from reservoir 16 towards reservoir 18. A sample may be introduced into
25 inlet 12a by means known to those skilled in the art, such as by gravity or by electrokinetic injection. Such sample would be caused to separate in tube 12 due to electrophoresis. In addition, the interaction between the sample components (which may be uncharged or neutral electrically) and the chromatographic particles 6 in the
30 glass matrix 13' causes chromatographic separation of the components as well.

 Instead of, or in addition to, the use of electrical potential to cause fluid flow in column 12, the fluid flow can also be caused by applying a pressure
35 differential along the column 12, which, in addition to or instead of the electric field applied along the

column, causes fluid flow from reservoir 16 to reservoir 18. This can be performed by using an enclosed reservoir 16 except for an inlet 16a, through which gas pressure is applied as shown in Fig. 4. The gas pressure may be supplied by means of a pump (not shown) for example. The gas pressure applied through inlet 16a causes buffer 14 to be pushed downward and the buffer to flow into the inlet 12a and column 12 to reservoir 18. Instead of using a pump to apply a pressure differential in column 12, a pressure differential may also be applied by raising the column inlet 12a to a position 12a' at an elevation higher than the outlet end 12b of the column, as shown in dotted lines in Fig. 4. Thus, as shown in dotted lines, the new positions of the inlet 12a' of the column, of reservoir 16' containing buffer 14' and electrode 30' immersed in the buffer in reservoir 16' are all at elevations above the outlet end 12b of the column. Alternatively, instead of raising the inlet end 12a, the same goal can be achieved by lowering outlet end 12b of the tube, by lowering reservoir 18, electrode 30 in such reservoir and end 12b. Or, the pressure in reservoir 18 can be reduced by means of a pump (not shown) to one below that of reservoir 16 to achieve a pressure differential between ends 12a, 12b, and to create fluid flow in tube 12.

In the embodiment of Fig. 2, only a portion of the column 12 is filled with the porous glass with chromatographic particles embedded therein, and the remaining portion of the column is not filled with the porous glass. In an arrangement similar to that in prior detection schemes, the separated sample components may be detected optically, such as by laser induced fluorescence as described in U.S. Patent No. 4,675,300 in a transparent column portion 12t that is transparent to radiation and downstream from glass 13' as shown in Figs. 2 and 4. As shown in Fig. 2, the portion 12t does

not contain any of the chromatographic particles present in glass matrix 13' and transmits radiation without significant scattering, such as UV or visible light. The sample components passing through such portion 12t
5 may be illuminated by means of radiation along arrow 50 to induce fluorescence, where the induced fluorescence may be detected by means of a detector 17 preferably placed away from the path of radiation 50. This is shown schematically in Fig. 4, where a detector 100
10 detects the separated sample components at location 12c. When detector 100 detects the sample components by means of laser induced fluorescence, the sample components may need to be first tagged by means of a fluorophore and detector 100 includes a laser source as well as a
15 photodetector.

In order to facilitate the evaporation of the volatile solvents in mixture 13 in Fig. 1, the tube 12 containing mixture 13 may be placed in an oven chamber 150 as shown in Fig. 5. Tube 12 is supported by a
20 pedestal 152 in the oven chamber and heat is applied by a heating coil 154 through which a current is passed by means of a power supply 156. To further facilitate the evaporation of volatile solvents, the gas pressure in oven chamber 150 is reduced by means of a pump 160.
25 While both heating and reduction of gas pressure may be used together to accelerate the evaporation of volatile solvents, heating may be used without pressure reduction and vice versa; all such variations are within the scope of the invention. When the volatile solvents have
30 evaporated, mixture 13 then forms a porous sol-gel glass that is attached securely to the inner walls of tube 12, with the chromatographic particles 6 embedded therein. It has been found that in some instances, a hardened porous sol-gel glass may form in about 24 hours.

35 To achieve a more uniform distribution of the chromatographic particles in the sol-gel, it may be

desirable to agitate the mixture 13 when the volatile components are escaping from the mixture. This can be done for example by rotating the tube along arrows 158, shaking the tube along arrows 162, or supplying
5 ultrasonic pulses along arrows 164 from an ultrasonic source (not shown) as shown in Fig. 5 in a manner known to those skilled in the art.

With this monolithic packing method, chromatographic materials that are charged and uncharged
10 in nature can be embedded into the sol-gel matrix. Different functionalized/derivatized sol-gel precursors can be used to prepare sol-gel glasses with different physical properties, such as pore size and surface charge. The pore size may be selected by choosing an
15 appropriate sol-gel precursor. For example, to obtain larger pores, tetramethylorthosilicate may be used as the precursor instead of tetraethylorthosilicate indicated above.

Mixture 13 may be prepared as follows. Micron or
20 submicron sized chromatographic particles, such as octadecylsilica may be added to a solution of water, alcohol, acid (optional) or base (optional) and a metal oxide source. The metal oxide source may include a silicate, titanate or zirconate. The resulting solution
25 or mixture 13 formed is injected by syringe into a column and heated overnight. No pre-fabricated frits is required.

Instead of using a syringe to inject the mixture 13 into a channel as described above in reference to Fig.
30 1, the mixture 13 may be introduced by means of a pressure differential as illustrated in Fig. 6, which is a cross-sectional view of a tube 12 along a longitudinal axis. As shown in Fig. 6, a pressure differential is applied between the inlet end 12a and outlet end 12b,
35 such as by means of a pump (not shown), in order to introduce a mixture 53 of water, alcohol, acid

(optional) or base (optional) and a metal oxide source, without chromatographic particles embedded therein, followed by a mixture 13 of chromatographic particles with a solution such as solution 53, which is again
5 followed by solution 53 to fill tube 12. Solution 53 and mixture 13 are then treated in the manner described above in reference to Fig. 5 to remove the volatile components and to form a porous sol-gel throughout the column 12. Only the section 12d of the tube containing
10 the porous sol-gel glass with chromatographic particles embedded therein is used for chromatographic separation. The remaining sections of the tube next to section 12d contain a porous sol-gel glass containing no chromatographic particles and, therefore, do not scatter
15 light used for detection, so that the scheme illustrated in Fig. 4 above may be used for detecting separated sample components in a similar manner employing the column of Fig. 6 that has been filled by a porous glass. The column 150 of Fig. 6 is advantageous in that the
20 entire tube is filled by the porous sol-gel glass so that there will no significant pressure differential between the fluid in the porous glass in section 12d and that in the porous glass in the remaining sections of the tube. This enhances separation performance.

25 The pressure differential between the inlet and outlet ends 12a, 12b may be created in many ways, such as by pushing (i.e. by increasing pressure) the mixtures 53, 13 into the inlet end 12a, or by reducing the gas pressure at outlet end 12b to draw in solution 53 and
30 mixture 13 as described (since such solution and mixture are under the higher atmospheric pressure at end 12a), by means of a pump (not shown). The inlet end 12a may also be placed at a higher elevation compared to outlet end 12b so that solution 53 and mixture 13 may be
35 introduced by hydrostatic pressure differential.

The packed "fritless" column columns 12 will facilitate the analysis of complex mixtures that may contain charged and/or uncharged compounds. The separation of a mixture of uncharged organic compounds has been demonstrated using a column packed in this manner. Advantages of the disclosed method include (i) easy and rapid injection of the hydrolysis reaction solution into the column, (ii) the elimination of inlet and outlet frit fabrication, (iii) incorporation of charged or uncharged chromatographic materials in the sol-gel matrix, (iv) UV transparency of the sol-gel glass, (v) potential for automation of many samples, and (vi) potential for large-scale preparative use. This results in a total column preparation time of approximately 24 hours and avoids the use of high pressures for post-column conditioning. The use of high voltages is also avoided during column preparation. This system is superior to both the electrokinetic and slurry packing methods.

Instead of using a sol-gel process as described above, other types of polymerization processes may be used, such as that described in the article "Macroporous Polyacrylamide/Poly(ethylene glycol) Matrixes as Stationary Phases in Capillary Electrochromatography," by Anders Palm and Milos V. Novotny, *Analytical Chemistry*, Vol. 69, No. 22, November 15, 1997, pp. 4499-4507; or the articles by P.G. Righetti, B.C.W. Brost and R.S. Snyder, in *J. Biochem. Biophys. Methods*, 1981, No. 4, pp. 347-363, and by P.G. Righetti, S. Caglio, S. Saracchi and M. Quaroni in *Electrophoresis*, 1992, No. 13, pp. 587-595. As described in these three articles, a porous matrix may be formed by polymerizing a solution of a monomer and a cross-linking reagent or initiator. If said solution is mixed with chromatographic particles and such mixture is used instead of mixture 13 to form the porous matrix with chromatographic particles

embedded therein, such matrix may also be used in a separation column for separating a sample into its components in the manner described above. When such a mixture is polymerized, the matrix forms a secure bond to the inner wall of the separation channel so that no frit is necessary to keep the matrix in place. The pores formed are big enough to permit diffusion species but small enough to prevent significant leaching of the chromatographic particles trapped therein. The porous matrix without the particles is transparent to radiation so that the configuration of Fig. 6 described above may be used, where the entire tube is filled with the porous matrix but only a section of the matrix is embedded with chromatographic particles. In this manner, the separated components may be detected downstream from the particles by a detector in a known manner, such as by means of laser induced fluorescence detection. The above-described method for introducing the mixture 13 may also be used for introducing a mixture of the particles with other types of monomers and cross-linking reagents (optional), such as acrylamide or ethylene glycol and an optional base or acid acting as a crosslinking reagent.

A porous matrix (whether or not embedded with particles) may be formed by heating or supplying radiation to a solution of a monomer such as acrylamide or ethyleneglycol and a cross-linking reagent (optional) such as a base or acid to form a macroporous polyacrylamide or poly(ethylene glycol) matrix. The polymerization is achieved thermally or by photochemistry. In reference to the article by Palm and Novotny, since chromatographic particles are used in this invention for sample separation, there is no need in this invention to include alkyl ligands as described in the Palm and Novotny article. Polymerization techniques different from the above may also be used for

forming the porous matrix; such and other variations are within the scope of the invention.

While the invention has been described above by reference to various embodiments, it will be understood
5 that different changes and modifications may be made without departing from the scope of the invention, which is to be defined only by the appended claims and the equivalents thereof.

WHAT IS CLAIMED IS:

1. A separation column comprising:
a separation channel having a channel wall; and
a separation medium in the channel, said medium
5 including a porous matrix attached to the channel wall
and chromatographic particles embedded in the matrix
forming a packed channel, said channel having no frit
therein.
2. The column of claim 1, said column having an
10 internal dimension in the range of between 5 and 5,000
microns.
3. The column of claim 1, said channel having a
elongated cross-section.
4. The column of claim 1, said column having a
15 first portion that is filled with said separation medium
and a second portion adjacent to said first portion that
transmits radiation.
5. The column of claim 4, said second portion
does not contain said separation medium.
- 20 6. The column of claim 1, wherein said matrix has
pores therein large enough for passage of neutral and
charged species but too small for passage of the
chromatographic particles.
7. The column of claim 1, said particles being
25 larger than 0.2 micron in dimensions.
8. The column of claim 1, said particles being a
mixture of different types of particles of different
sizes.

15

9. The column of claim 1, said matrix including a sol-gel glass.

10. The column of claim 9, wherein said glass includes a silicate, titanate or zirconate.

5 11. The column of claim 1, said column being filled with a porous matrix, said matrix having embedded therein chromatographic particles in only a section of the column.

12. The column of claim 1, said matrix including
10 a polyacrylamide or polyethylene glycol.

13 An apparatus for separating a sample into its components, comprising:

a separation channel having a channel wall;

a separation medium in the channel, said medium
15 including a porous matrix attached to the channel wall and chromatographic particles embedded in the matrix, forming a packed channel, said channel having no frit therein; and

means for causing a fluid containing a sample
20 present in the channel to flow and the sample to separate.

14. The apparatus of claim 13, said channel being a capillary having an internal dimension in the range of between 5 and 300 microns.

25 15. The apparatus of claim 13, said channel having a first section that is filled with said separation medium and a second section adjacent to said first portion that transmits radiation.

16

16. The apparatus of claim 15, said second section does not contain said separation medium.

17. The apparatus of claim 16, said second section containing a porous matrix not embedded with chromatographic particles.

18. The apparatus of claim 17, said channel filled with a porous matrix.

19. The apparatus of claim 15, said causing means causing the sample to pass through the second section, said apparatus further comprising:

means for applying radiation to the sample in the second section; and

means for detecting radiation from the second section to detect components of the sample passing through the second section.

20. The apparatus of claim 13, said causing means applying an electric field along the channel, so that the sample separates by electrophoresis and chromatography.

21. The apparatus of claim 13, said causing means applying a pressure differential along the channel, so that the sample separates by chromatography.

22. The apparatus of claim 13, said causing means applying both an electric field and a pressure differential along the channel, so that the sample separates by electrophoresis and chromatography.

23. The apparatus of claim 13, wherein said matrix has pores therein large enough for passage of neutral

and charged species but too small for passage of the chromatographic particles.

24. The apparatus of claim 13, said particles being larger than 0.2 micron in dimensions.

5 25. The apparatus of claim 13, said matrix including a sol-gel glass.

26. The apparatus of claim 25, wherein said glass includes a silicate, titanate or zirconate.

10 27. The apparatus of claim 13, said column being filled with a porous matrix, said matrix having embedded therein chromatographic particles in only a section of the column.

28. The apparatus of claim 13, said matrix including a polyacrylamide or polyethylene glycol.

15 29. A method for making a separation column, comprising:

introducing a mixture of chromatographic particles and a solution of a monomer and a cross-linking reagent into a separation channel, said channel having a wall;

20 causing the mixture to form a porous matrix attached to the channel wall with said particles embedded therein.

30. The method of claim 29, said introducing employing a pressure differential.

25 31. The method of claim 30, wherein said employing uses a syringe.

32. The method of claim 29, said channel being a capillary, wherein said introducing injects said mixture into said capillary.

33. The method of claim 29, wherein said causing
5 includes heating or supplying radiation to the mixture until it gels.

34. The method of claim 29, wherein said causing
includes reducing gas pressure in a chamber containing
the mixture, to remove volatile components from the
10 mixture.

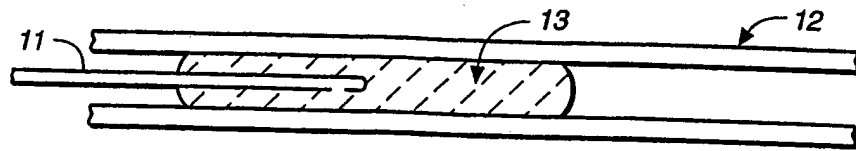
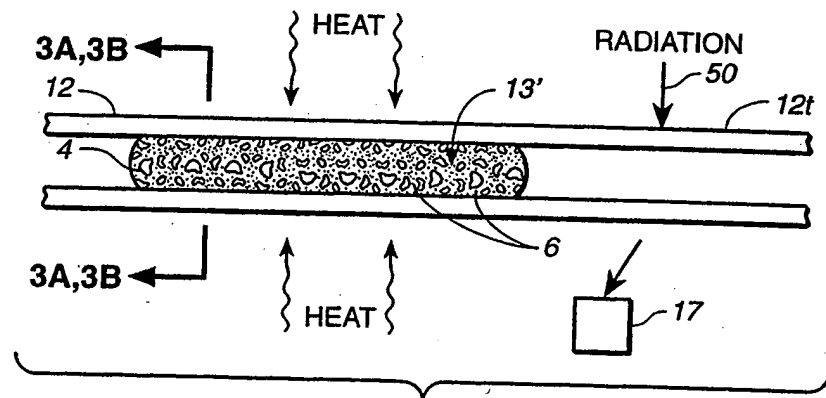
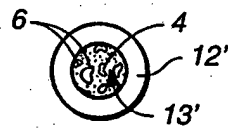
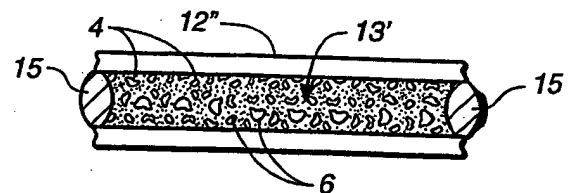
35. The method of claim 29, wherein said causing
includes agitating the mixture so that the particles are
more uniformly distributed in the matrix.

36. The method of claim 35, wherein said agitating
15 includes rotating or vibrating the channel, or applying
ultrasonic pulses to the channel.

37. The method of claim 29, wherein said
introducing introduces a solution of water, alcohol and
a metal alkoxide into the channel.

20 38. The method of claim 29, wherein said
introducing introduces a solution of a acrylamide or
ethylene glycol and a base or acid into the channel.

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**FIG._1****FIG._2****FIG._3A****FIG._3B**

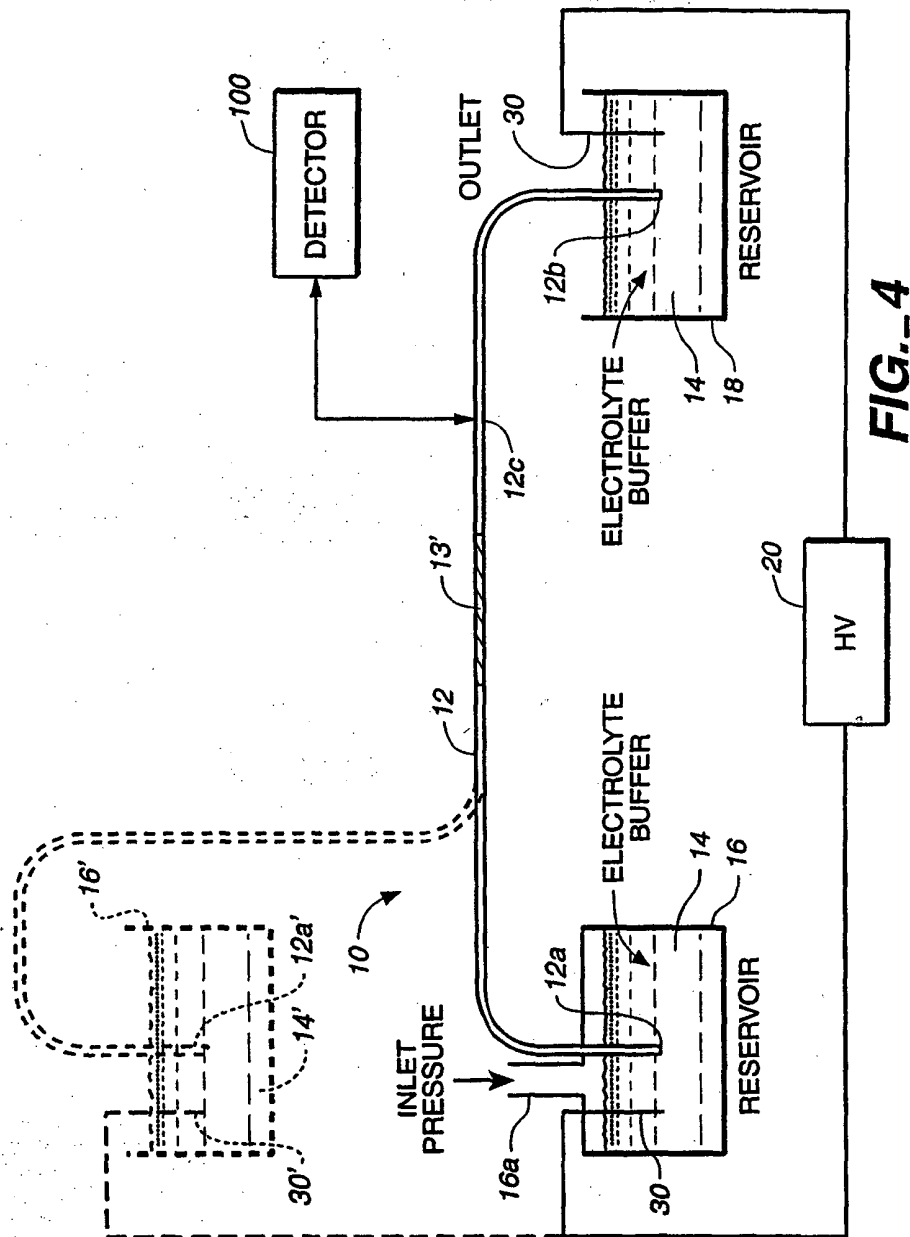
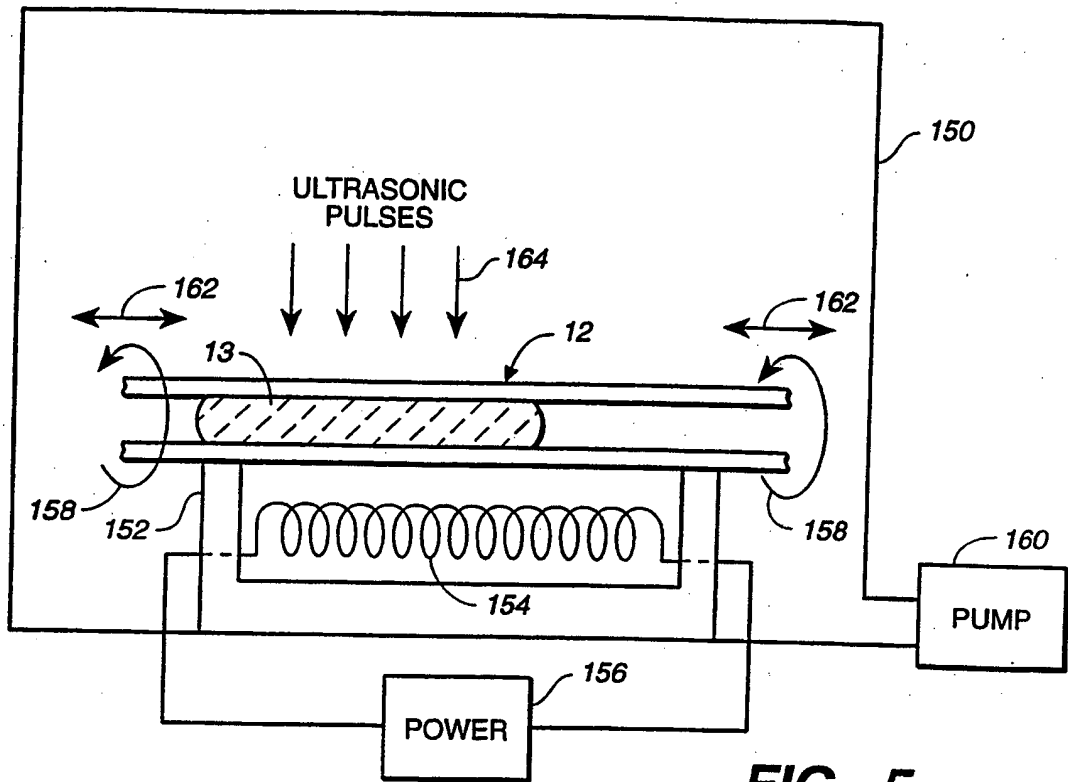
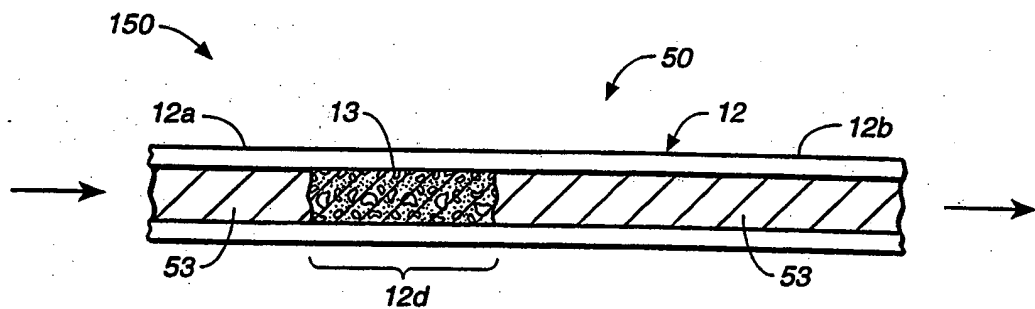


FIG. 4

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**FIG._5****FIG._6**

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